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Title: Diurnal rhythm and salivary electrolyte

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In the October, 2017 issue of Archives of Oral Biology a paper from Bel'skaya et al. was published describing an investigation into the influence of a diurnal rhythm on salivary mineral composition (Bel'skaya, Kosenok, & Sarf, 2017). Saliva samples were collected at 3 hr intervals over a 24 hr period and pH, calcium, phosphorus, sodium and potassium concentrations were assayed. Following publication, the Editors received a letter from Professor Colin Dawes, who has published seminal research papers on the influence of diurnal rhythms on saliva mineral composition, expressing a number of concerns regarding the paper.

In the present editorial we have highlighted the major concerns raised by Professor Dawes and in the interests of balance, have given the authors an opportunity to respond. The purpose of this editorial is two-fold. Firstly, it clarifies some aspects of the published paper and enables the reader to make a judgement based on the concerns raised and the answers given. Secondly, it highlights some aspects of salivary research that require the attention of those intending to undertake such studies.

Major concerns expressed by Professor Dawes:

1. The readers are not told specifically whether the saliva was unstimulated or stimulated, although it was probably unstimulated. The timing of meals in relation to the saliva collection times is also not given. This is important, since the composition of saliva collected within one hour of a meal may be changed (Dawes & Chebib, 1972).
2. The saliva samples were centrifuged prior to assay but as we are told only the revolutions per minute of the centrifuge and not its radius, it is not possible to calculate the centrifugal force applied in gravitational units.
3. All the assays, including measurement of the pH, were apparently done using a capillary electrophoresis system, with quantification of different components at different wavelengths. The description of the apparatus is incomprehensible, at least to this reader. Accurate measurement of pH requires that saliva not be exposed to the atmosphere, which causes a loss of CO_2 and a rise in pH. As no such precautions were mentioned, the pH values are suspect.
4. Prior to assay of various inorganic components of the saliva, salivary protein was said to be "deposited" (presumably precipitated) with 10% trichloroacetic acid. However, although this reagent precipitates most plasma proteins, it does not effectively precipitate most salivary proteins (Dawes, 1965).
5. The study began at 3 a.m. and saliva collections continued every three hours until midnight of the same day. However, in the four Figures, the midnight values have been duplicated at midnight of the previous day.
6. On the last two lines of page 287, it states that "higher salivary flow rates were associated with lower levels of sodium ions". This is the opposite of what would be expected, as the sodium concentration in saliva normally increases with flow rate.
7. The salivary potassium concentrations average about 9.5 mmol/L, which is about half the value expected from the literature.
8. The mean values at different time points for men alone, women alone and men + women in Figures 1 – 4, are all connected by beautifully-shaped curves which pass through every point. However, the derivation of these curves is not described.

9. On page 289, line 6 of column 1, it is written “The mineralizing function of saliva is carried out due to its supersaturation with Ca^{2+} and HPO_4^{2-} ions.” However, saliva can not be saturated with individual ions, only with respect to a mineral.
10. On page 290, line 5 of column 1, it is written “This finding can be explained through higher solubility of calcium phosphates at lower acidity of the medium.” This is nonsense, as the solubility of all calcium phosphates increases as the pH falls and decreases as it rises.

Responses from authors to comments:

1. Study plan included a collection of non-stimulated saliva not earlier than 1.5 hours after a meal.
2. Centrifugation was carried out at the acceleration (Relative Centrifugal Force) of 8610 g.
3. The capillary electrophoresis technique is widely described in the literature (Demkowska, Polkowska, & Namieśnik, 2008; Guo et al., 2016; Mori et al., 2016; Mori, Yamamoto, Kaseda, Yamada S, & Itabashi, 2012); this article provides an in-depth description of conditions for analysis and preparation of the samples (Bel’skaya, 2017). With regard to the pH measurement, saliva was collected into test tubes with tightly screwing caps, and pH was measured immediately after the opening of the tubes. Saliva samples were stored at a temperature of 2-8°C; all analyses were performed within 24 hours after collection.
4. Deposition of proteins with 10% trichloroacetic acid was carried out in order to prevent contamination of the capillary during the recording of electropherograms of the samples. Since protein composition of saliva was not analyzed, it could not affect the results and their interpretation.
5. There was no duplication of values, these data are the same and are shown in the graph.
   The experiment was repeated for 3 days, the values obtained at the same time are almost the same. The error does not exceed 10%.
6. Secretion of saliva is a two-step process: the first stage involves acini, the second – salivary ducts. Acini secrete primary secret that contains ptyalin and/or mucus in a solution with the concentration of ions that is not much different from the concentration of ions in extracellular fluid. As the primary secretion progresses through channels, sodium ions are actively reabsorbed by all salivary ducts and potassium ions are actively secreted in exchange for the sodium, so the concentration of sodium ions in saliva significantly reduces, while the concentration of potassium ions increases. In this article, we provided experimentally obtained fact that the sodium concentration and the salivation rate change in opposite directions. We did not touch upon this subject in our work, but a reduction of sodium level and an increase of potassium concentration may not only depend on the salivation rate (Jirakulsomchok & Schneyer, 1987). We hope to investigate this question more in our further studies.
7. In the literature, the concentration of potassium in saliva varies in a wide range. It may be due to the different conditions of collection and storage, as well as the modes of centrifugation and separation of a sediment that contains intracellular potassium from damaged cells giving the excessive concentrations.
8. Curves were plotted using interpolation method with Bezier curves (R Studio 3.3.0).
9. Basing on the theory of micellar structure of saliva $\{[m(Ca_3(PO_4)_2]nHPO_4^{2-}(n-x)Ca^{2+}\}^{2x-}xCa^{2+}$, the saliva is over-saturated with Ca^{2+} and HPO_4^{2-} in relation to the calcium phosphate.

10. The solubility of calcium phosphates decreases with increasing pH of the medium. This fact is confirmed by thermodynamic calculations, what is written on page 290, row 5, column 1. Here the solubility of calcium phosphates increases along with decreasing pH of the medium. We wanted to reflect this idea in our article. We want to say that this finding can be explained by higher solubility of calcium phosphates at lower pH of the medium.

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